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RESEARCH ARTICLE

Creutzfeldt astrocytes may be seen in IDH-wildtype glioblastoma and retain expression of DNA repair and chromatin binding proteins

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Abstract

Astrocytes with multiple micronuclei ("Creutzfeldt cells") in a brain biopsy are classically associated with demyelinating disease. However, glioblastoma may also have prominent Creutzfeldt astrocytes, along with granular mitoses. Therefore, Creutzfeldt cells may raise the diagnostic dilemma of high-grade glioma vs tumefactive demyelination. While cases of glioblastoma (GBM) with Creutzfeldt astrocytes have been reported, their clinicopathologic spectrum and genetic features are not understood. Studies have proposed that micronuclei in Creutzfeldt cells are a consequence of DNA damage, or may be susceptible to DNA damage and chromothripsis, but their biology in the context of glioblastoma remains unclear. Based on a challenging index case of GBM with mild hypercellularity, Creutzfeldt astrocytes, and granular mitoses on biopsy, we searched our archives for additional cases with similar histopathologic features. We identified 13 cases, reviewed their clinico-radiologic and pathologic features, and examined them for recurrent genetic alterations via NGS (9 cases) and for evidence of DNA damage by immunohistochemistry for DNA repair and chromatin remodeling proteins. We found that Creutzfeldt cell-rich GBMs were IDH-wildtype with no recurring genetic alterations. To test our hypothesis that micronuclei demonstrate loss of DNA repair or chromatin remodeling proteins, we examined the expression of various proteins (MDM2, p53, MLH1, MSH2, PMS2, MSH6, ATRX, INI1, SATB2, Ki67, pHH3) in Creutzfeldt cell rich-GBM. There was intact expression of DNA repair and chromatin remodeling proteins, with accumulation of p53 and reduced MDM2 expression within micronuclei. In contrast, granular mitoses showed pHH3 expression, confirming these cells are undergoing mitotic division, with no accumulation of p53 and reduced expression of DNA repair proteins. Our results emphasize that Creutzfeldt cells are part of the morphologic spectrum of IDH-wildtype glioblastoma. We did not find a role for DNA damage in the generation of Creutzfeldt cells, as both DNA repair and chromatin remodeling protein expression was retained in these cells.

INTRODUCTION

Creutzfeldt astrocytes are astrocytes containing multiple micronuclei, which are of smaller size than the nuclei of adjacent tumor cells. Creutzfeldt astrocytes are often accompanied by cells with minute chromosome bodies ("granular mitoses") and these were originally described by the German neuropathologist Creutzfeldt in patients with a clinical diagnosis of multiple sclerosis (4). This original description led to the association of Creutzfeldt astrocytes and granular mitoses with demyelinating disease (4, 23), and the presence of Creutzfeldt astrocytes in a biopsy remains a strong indicator of a demyelinating, rather than neoplastic, process. However, a tumor cell population with micronuclei and granular mitoses can also occur in glioblastoma (GBM), and tumefactive demyelination and high-grade glioma may not always be distinguished on imaging grounds alone (9, 10, 12, 14). Cases with a possible "collision" of inflammatory (tumefactive) demyelination and high-grade glioma are also reported (17). Although in many

cases the clinico-radiologic and histologic features are sufficient to distinguish GBM and demyelinating disease, there is overlap and the misdiagnosis of demyelination as glioblastoma (or vice versa) is a well-recognized pitfall in the field of surgical neuropathology (6, 23).

Creutzfeldt astrocytes and granular mitoses, despite their clinical relevance, have received fairly limited attention in the neuropathology literature. The micronuclei likely originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division (1, 3, 7), but little is known about the causes and consequences of micronuclei formation. Exemplifying the diagnostic difficulties posed by these cells, we recently encountered a challenging case of a 78-year-old male presenting with a fairly circumscribed right temporo-parietal mass on magnetic resonance imaging (MRI) that demonstrated slow radiologic progression across multiple pre-operative imaging studies (2). Minimal mass effect was present and enhancement was heterogeneous. Biopsy was eventually performed, which showed mildly hypercellular tissue with atypical astrocytes, macrophages, prominent Creutzfeldt cells and granular mitoses, focal endothelial proliferation, a proliferation index ranging from 1% to 10% and no necrosis. A panel of molecular studies was noncontributory, revealing no mutations in *IDH*, retention of ATRX protein, no *PTEN* or *EGFR* alterations and no mutations in genes involved in gliomagenesis by a 50-gene panel (eg, *BRAF, CDKN2A, PIK3CA* and *TP53*). Nonetheless, consensus review led to a diagnosis of glioblastoma, IDH-wildtype, WHO grade IV, leading to subsequent gross total excision of more cellular tumor, including foci with subpial spread.

Although Creutzfeldt astrocytes may be seen in non-demyelinating neuropathology specimens, as exemplified in the case above, their etiology remains unknown, particularly in the setting of malignancy. Micronuclei have generally been considered a consequence of DNA damage (20). These micronuclei may affect basic nuclear functions, such as DNA repair and replication, and thus generate massive damage in the chromatin and contribute to DNA damage and chromothripsis (ie, extensive genomic rearrangements restricted to one or few chromosomes) (11, 19, 20, 22, 24). Chromothripsis involving chromosome 6 has been recently reported as an infrequent finding in glioblastoma, but may be associated with aggressive behavior (16).

Our experience with the index case described above motivated us to search for additional cases of GBM in which Creutzfeldt astrocytes and/or granular mitoses were a frequent component. Given that glioblastomas with abundant Creutzfeldt astrocytes and granular mitoses are relatively rare, we hypothesized that the cohort of cases would share similar clinico-radiologic features, and possibly, recurrent genetic alterations explaining their unusual morphology. We also hypothesized that micronuclei in Creutzfeldt cells would demonstrate loss of DNA repair or chromatin remodeling proteins, consistent with the concept that these cells are a consequence of DNA damage (20). To test these hypotheses, we identified 13 GBMs with Creutzfeldt astrocytes and granular mitoses ("Creutzfeldt cell-rich GBM"), explored their mutational profile and assessed the expression of proteins involved in DNA repair (MLH1, MSH2, PMS2, MSH6), chromatin remodeling (ATRX, INI1, SATB2) and the DNA damage response (p53, MDM2).

METHODS

Cases

This study was done in compliance with the institutional review board of Houston Methodist Hospital (Pro00014350) and UT-MD Anderson Cancer Center (PA17-0216). We searched the electronic archives of the pathology department of both institutions (2006–2016) for glioblastoma cases in which the presence of Creutzfeldt astrocytes or granular mitoses was noted in the pathology report. A total of 13 cases were identified. Histologic features, including the presence of atypical mitoses, granular mitoses, Creutzfeldt astrocytes, perivascular lymphocytes, vascular proliferation, necrosis and microthrombi, were reviewed.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using formalin-fixed, paraffin-embedded (FFPE) tumor tissue sections on an automated Bond Max (Leica Microsystems, Buffalo Grove, IL). The following antibody clones were utilized: P53 (clone DO-7, 1:100, Dako), Ki67 (clone MIB-1, 1:100, Dako), MDM2 (clone 1B10, 1:100, Leica/Novocastra), MSH2 (clone FE11, 1:100, Calbiochem), MLH1 (clone G168-728, 1:300, Cell Marque), MSH6 (clone 44, 1:300, BD Biosciences), PMS2 (clone A16-4, 1:125, BD Biosciences), pHH3 (catalog # 06-570, 1:400, Milipore), INI1 (Clone 25, 1:50, BD Biosciences), BAP1 (clone C-4, 1:150, Santa Cruz), ATRX (catalog # HPA001906, 1:400, Sigma) and SATB2 (clone CL0276, 1:50, Sigma). IHC interpretation was performed by a board-certified neuropathologist (L.Y.B.) in all cases.

DNA extraction from FFPE tissue

DNA was extracted from FFPE tumor samples. Briefly, H&E-stained tissue sections were reviewed by a pathologist who estimated the tumor percentage and identified the area for dissection. Unstained 10-mm thick tissue sections were deparaffinized and tumor tissue was manually dissected. Specimens with greater than 50% tumor in the circled area were included in the study. Genomic DNA was extracted using a PicoPure DNA extraction kit (Arcturus, Mountain View, CA) and purified using an Agencourt AMPure XP kit (Agencourt Biosciences, Beverly, MA) according to the manufacturer's instructions. A Qubit DNA high-sensitivity assay kit (Life Technologies) was used to quantify the extracted gDNA.

Next generation sequencing

Next generation sequencing was performed in our laboratory using an amplicon-based, 50-gene mutation hotspot test according to the manufacturer's instructions (Ampliseq Cancer Hotspot Panel version 2; Life Technologies, Grand Island, NY). The following genes were tested: *ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53* and *VHL*. A minimum of 100× coverage was required at a given base for the interpretation of a wild-type or

Table 1. Clinicopathologic characteristics of glioblastoma with Creutzfeldt astrocytes.

	Dx	Age	Sex	Site	Mutations	Mitosis	Granular mitoses	Creutzfeldt astrocytes	Lymphocytes	VP	Necrosis	Microthrombi	Alive (A) or decreased (D)	Survival (days)
1	GBM	67	M	Right parietal	WT	Atypical	+	+	+	+	+	−	D	96
2	GBM	50	M	Left parietal	WT	+	+	+	+	+	+	+	D	824
3	GBM	74	F	Left temporal	PIK3CA p.His1047Arg	Atypical	+	+	−	+	−	−	Unknown	Unknown
4	GBM	69	M	N/A	WT	Atypical	+	−	−	+	+	+	D	Unknown
5	GBM	66	F	Right temporal	WT	Atypical	+	+	−	+	+	+	D	600
6	GBM	63	F	Right parietal	PTEN p.Arg130Gly, NF1 p. R897Y, ERBB2 p.R487Y	+	+	+	+	+	+	+	D	150
7	GBM	62	M	Left frontal	IDH-WT (sequencing limited to IDH1/IDH2)	+	+	+	−	+	+	+	D	268
8	GBM	79	M	Right occipital	PIK3CA p.Asn1044Lys ; PTPN11 p.Gly503Val	+	+	+	+	+	+	+	D	516
9	GBM	78	M	Right temporal	NOTCH1 p.P1442L, NF1 p.P2016L	+	+	+	+	+	+	+	D	417
10	GBM	45	M	Right parietal	N/A	+	+	+	+	+	+	+	A	>583
11	GBM	59	F	Left temporal	N/A	+	+	+	+	+	+	+	Unknown	Unknown
12	GBM	52	F	Right temporal	N/A	+	+	+	+	+	+	−	Unknown	Unknown
13	GBM	70	M	Left frontal	WT	+	+	+	+	+	+	+	D	477

variant call and the limit of detection of the assay was 10% (one mutant allele in the background of nine wild type alleles).

RESULTS

Clinical characteristics and histopathology

A total of 13 cases were identified (Table 1). The cases included 8 males and 5 females, with an age range from 45 to 79 years. Some cases showed areas with mild hypercellularity and atypical cells evenly distributed throughout the lesion, making the distinction from a reactive process difficult (Figure 1). The presence of perivascular lymphocytes and macrophages in some cases also contributed to the diagnostic difficulty conveyed in the initial pathology reports. However, all cases showed at least focal histologic features of high-grade infiltrating glioma, including vascular proliferation or necrosis, consistent with glioblastoma. Mitotic activity was present in all cases and atypical mitotic figures were identified in 4 of 13 cases. The presence of vascular microthrombi was noted in 10 of 13 cases and vascular proliferation was present in all cases. Perivascular lymphocytes were identified in 7 of 13 cases. Representative hematoxylin and eosin (H&E) images for 5 cases are shown in Figure 1 and representative images of Creutzfeldt cells and granular mitoses at higher magnification are shown in Figure 2. One observation that emerged from microscopic examination of all cases is that the Creutzfeldt cells and granular mitoses may occur focally and in clusters and not be evenly distributed throughout the tumor (Figure 1C).

Next generation sequencing results

We utilized a next generation sequencing assay that examines hotspot mutations in 50 cancer-associated genes (see "Methods" section). Out of the nine cases that were tested, mutations were detected in five cases (Table 1). Surprisingly, no mutations were identified in the remaining four GBMs. The *IDH1* and *IDH2* genes were examined in eight cases as part of the panel and by targeted sequencing of both genes in one case (for a total of 9 cases tested)—all cases were *IDH1/IDH2* wildtype. Two (of nine) cases showed *PIK3CA* mutations and another two cases showed *NF1* mutations (Table 1).

P53 and MDM2 expression

We examined the expression of p53 in tumor cells and in Creutzfeldt astrocytes by immunohistochemistry in 4 cases. Only one case showed diffuse strong expression of p53 protein in the tumor cells. However, there was strong expression of the p53 protein in the Creutzfeldt astrocytes (Figure 3) of all cases tested. In contrast, the granular mitoses did not express p53 protein. As MDM2 regulates p53 expression by inducing its degradation through the ubiquitin pathway, we examined the expression of MDM2 in Creutzfeldt cells and granular mitoses. Creutzfeldt cells did not show expression of MDM2, consistent with the high level of p53 protein detected in these cells. In contrast, MDM2 expression was detected in the granular mitoses (Figure 3).

pHH3 and Ki-67 expression in granular mitoses

Although the cells with granular chromatin are often interpreted as undergoing mitotic division based on morphologic features, we found no studies investigating the expression of proliferation markers in these cells. We found that granular mitoses in our cases expressed the proliferation antigen Ki67 and phosphorylated histone 3, a marker of mitotic division (Figure 3). Creutzfeldt astrocytes similarly expressed Ki67, but expression of pHH3 was not detected.

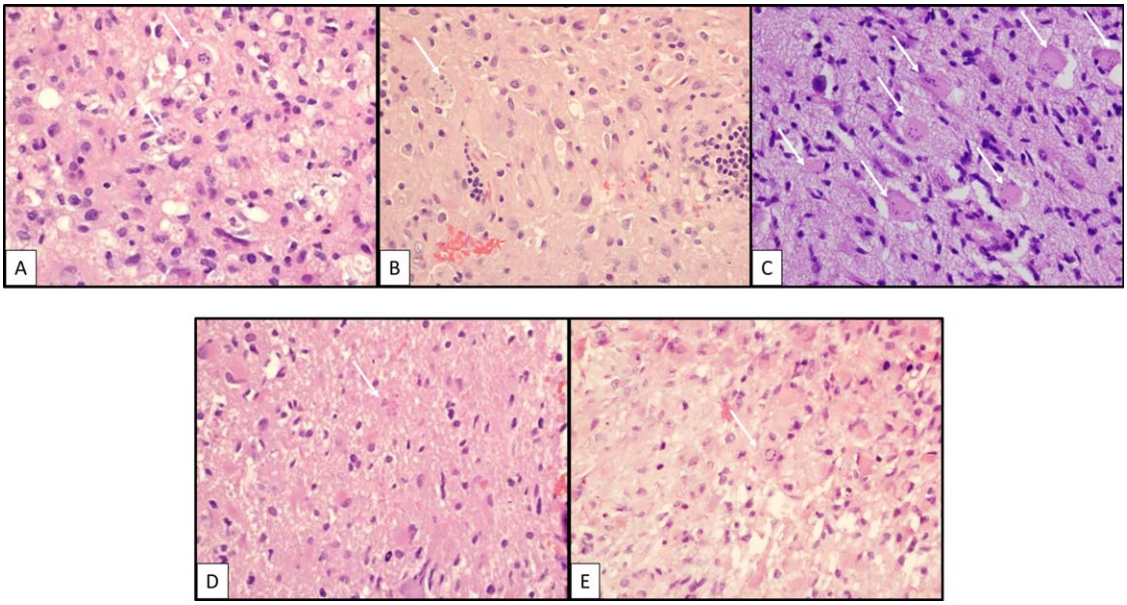

Figure 1. Examples of five different cases of glioblastoma with Creutzfeldt astrocytes and granular mitoses (indicated by white arrows). As depicted, in some instances, the histologic features make it difficult to distinguish a neoplasm from a demyelinating process and the increase in cellularity may be mild (panel D). Other cases may contain clusters of Creutzfeldt astrocytes (panel C). All images photographed at 100×.

Expression of chromatin remodeling proteins (SATB2, INI1, ATRX)

We examined the expression of chromatin remodeling proteins by immunohistochemistry in 2 cases with abundant Creutzfeldt astrocytes. Immunohistochemical studies revealed that Creutzfeldt astrocytes had normal expression of INI1 and retained nuclear expression of ATRX (Figure 4). Expression of SATB2 was weak-to-negative in both Creutzfeldt cells and granular mitoses

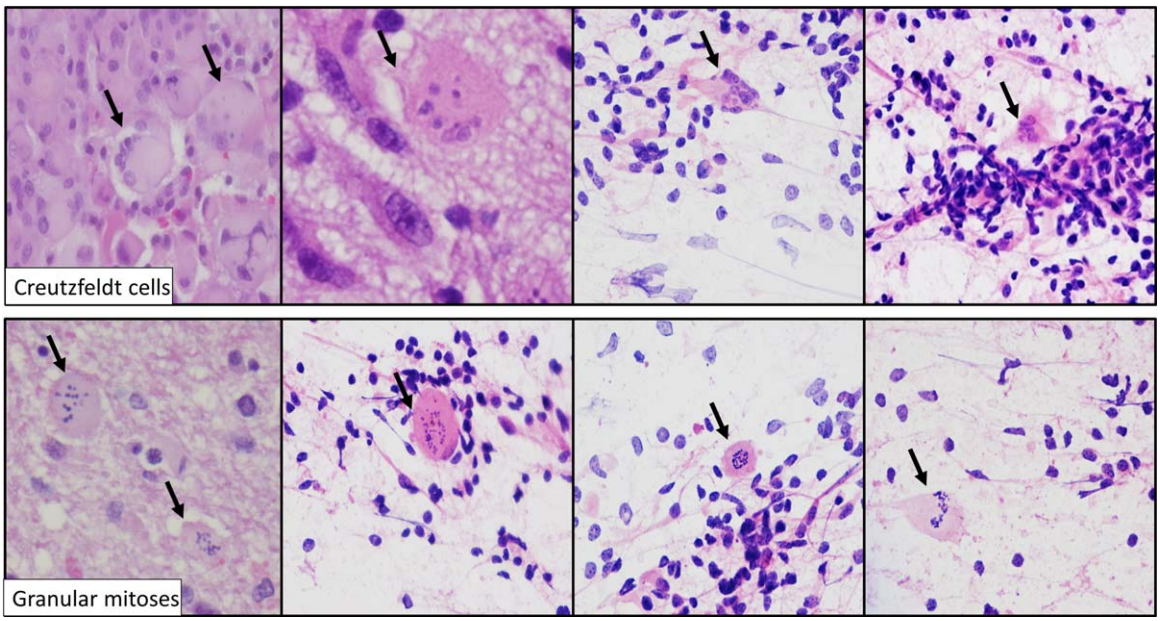

Figure 2. Creutzfeldt astrocytes (top row) and granular mitoses (bottom row) in glioblastoma in formalin-fixed paraffin-embedded (FFPE) tissue sections and in smear preparations. As shown here (top row), Creutzfeldt astrocytes are identified by their multiple micronuclei of varying sizes, which are typically smaller than the nuclear size in adjacent tumor cells. Granular, or "starbust," mitoses are also conspicuous and may be identified in both demyelinating processes and glioblastoma. All images were photographed at 200× with the exception of two left-most images in the top row and the left-most image in the bottom row, which were photographed at 400×.

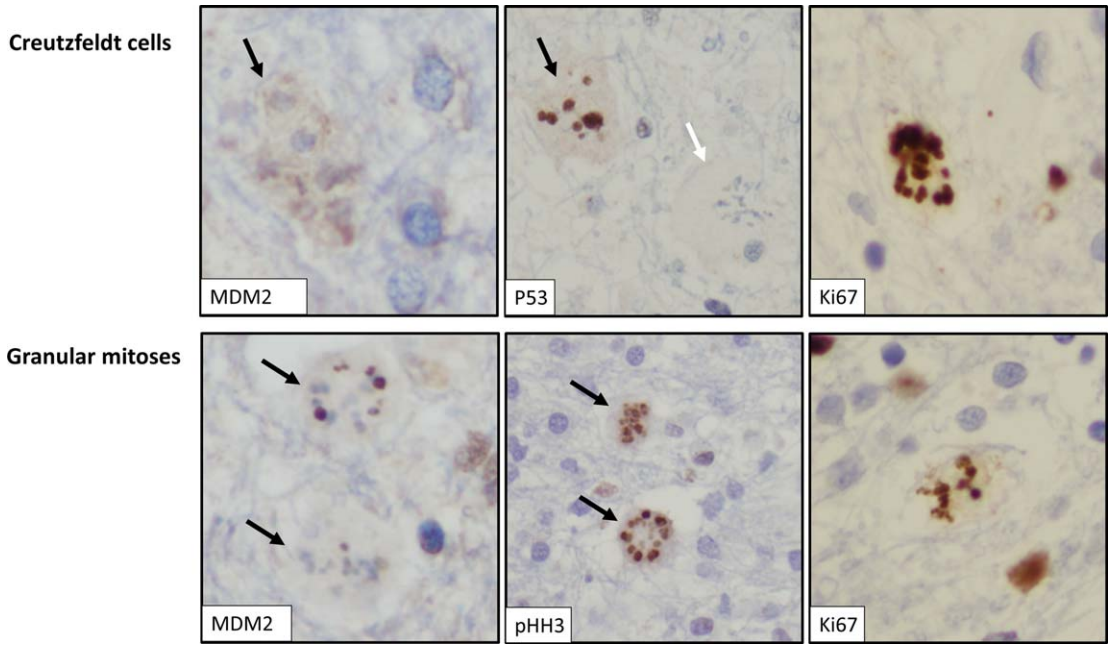

Figure 3. Creutzfeldt astrocytes express p53 protein (top middle panel) but not MDM2 (top left panel, black arrow). In contrast, granular mitoses do not express p53 (top middle panel, white arrow). Weak expression of MDM2 was noted in granular mitoses (bottom left panel, black arrows). Granular mitoses expressed pHH3 and Ki67 proteins (bottom middle panel, black arrows, and bottom right panel), consistent with the notion that these cells are in the active phase of the cell cycle (Ki67) and undergoing mitotic division (pHH3). Creutzfeldt astrocytes also express Ki67, indicating that the cells are in the active phase of the cell cycle, but not pHH3 (not shown). All images were taken at 400×.

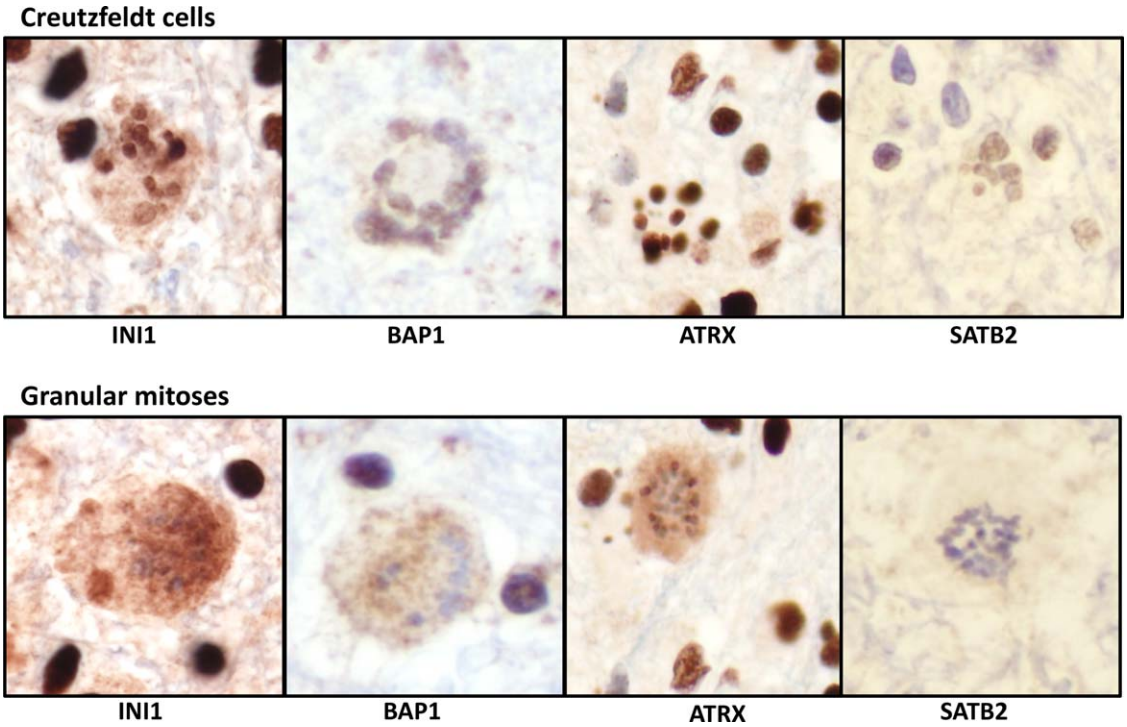

Figure 4. Expression of DNA-associated proteins in Creutzfeldt astrocytes (top row) and granular mitoses (bottom row) in glioblastoma. Note the normal expression of INI1, BAP1, ATRX and SATB2 in Creutzfeldt astrocytes. In contrast, the expression of INI1, BAP1 and SATB2 is downregulated in granular mitoses. ATRX protein is expressed in both cell types and appears to be associated with the condensed chromatin in granular mitoses. All images photographed at 400×.

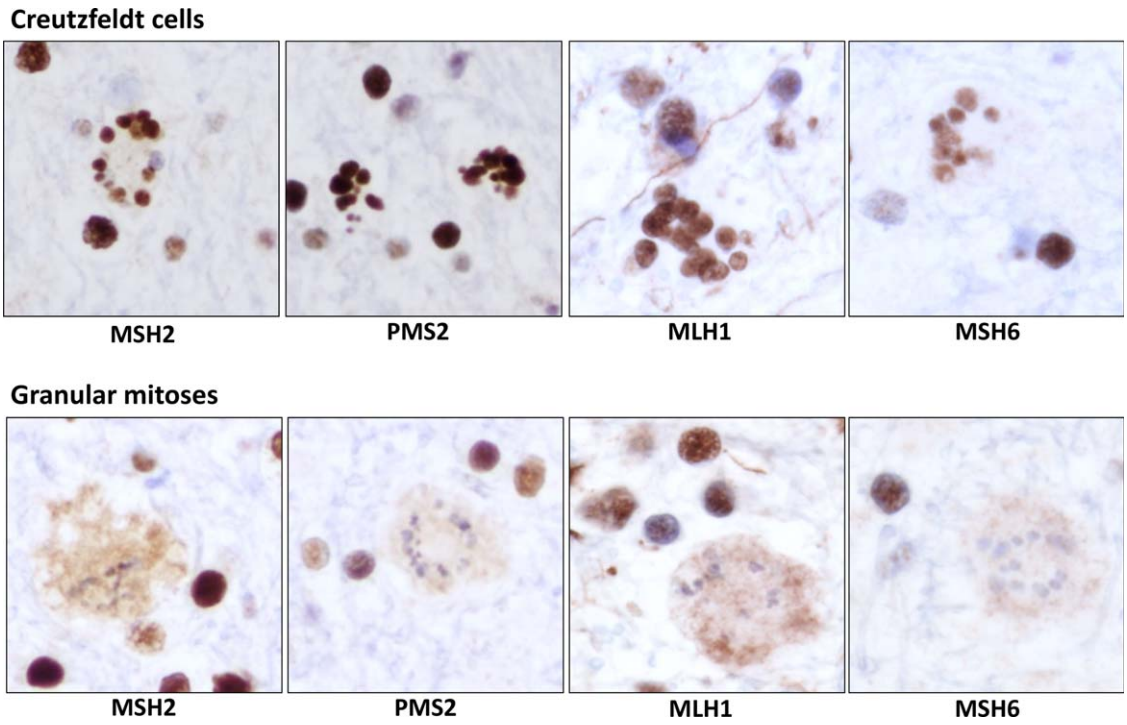

Figure 5. Expression of DNA mismatch repair proteins in Creutzfeldt astrocytes (top row) and granular mitoses (bottom row). Note the normal expression of MSH2, PMS2, MLH1 and MSH6 in Creutzfeldt astrocytes, but not in granular mitoses (bottom row). This shows that DNA repair proteins are normally expressed in Creutzfeldt astrocytes, but are downregulated during mitotic division.

(Figure 4), in contrast to strong expression displayed by normal neurons present in fragments of cortex adjacent to the tumor (data not shown). SATB2 expression was weak or negative in normal astrocytes and oligodendroglial cells.

Expression of DNA repair proteins (MLH1, PMS-2, MSH2 and MSH6)

Based on the hypothesis that Creutzfeldt astrocytes might have reduced levels of DNA repair proteins, contributing to the formation of micronuclei, we evaluated the expression of MLH1, PMS-2, MSH2 and MSH6 in Creutzfeldt astrocytes and granular mitoses by immunohistochemistry (Figure 5). The micronuclei in Creutzfeldt astrocytes showed normal expression of MLH1, PMS-2, MSH2 and MSH6. In contrast, the granular mitoses showed reduced-to-absent levels of these proteins (Figure 5).

DISCUSSION

To our knowledge, this is the first study of unusual glioblastomas enriched in hypertrophic astrocytes with micronuclei (Creutzfeldt cells) and granular mitoses, particularly with respect to the mutation profile of these tumors and the possible role of DNA damage and impaired DNA repair in the formation of the multinucleated cells. Several conclusions can be drawn from our study, although these are tempered by the small sample size. First, all glioblastomas studied occurred in patients older than 45 years of age and were *IDH*-wildtype—findings consistent with "primary" glioblastoma. Second, despite pathologic materials with mild hypercellularity in some cases, vascular microthrombi were a fairly consistent morphologic feature, which is known to be tightly correlated with the *IDH*-wildtype status in glioblastoma (21). Third, the outcome in all patients studied was consistent with that expected in *IDH*-wildtype glioblastoma, even in cases with fairly slow radiologic progression and apparent circumscription on MRI studies and intraoperatively. Fourth, no recurrent mutations were identified that would define this as a unique subtype of glioblastoma. Two tumors each harbored mutations in *PIK3CA* and *NF1*, which is consistent with prior studies indicating that these are common alterations in glioblastomas (13). Finally, Creutzfeldt cells and granular mitoses in GBM were confirmed to be in the active phase of the cell cycle by Ki67 and pHH3; however, no alterations in proteins involved in DNA repair or chromatin remodeling were identified in Creutzfeldt cells.

Creutzfeldt astrocytes contain an abnormal number of micronuclei, and in our cases showed elevated levels of p53 expression in the absence of *TP53* mutation. The p53 protein is the product of a tumor suppressor gene with the ability to bind damaged DNA and regulate the cell cycle, with normal cells having low levels of p53 expression. In the setting of DNA damage, p53 expression increases, with the goal of arresting cell cycle progression, activating expression of DNA repair proteins and preventing the replication of cells with damaged DNA. An association between chromothripsis and *TP53* mutation has been reported (15) and

micronuclei formation has been associated with accumulation of p53 in head and neck cancers (5). However, a link between *TP53* (or any other specific) mutation and Creutzfeldt astrocytes was not supported by the results of the present study.

Creutzfeldt astrocytes expressed Ki67, indicating they are in the active phase of the cell cycle (8), but expression of pHH3 was not detected, indicating they were not actively undergoing mitosis. In contrast, pHH3 expression in "granular mitoses" supports the idea that they are actively undergoing mitosis. As we observed normal expression of INI1 and ATRX proteins, our data fail to support the hypothesis that chromatin remodeling proteins are altered in Creutzfeldt astrocytes. We also observed intact expression of DNA repair proteins in Creutzfeldt astrocytes, which fails to support the hypothesis that DNA repair proteins are altered in these cells. In contrast, granular mitoses showed reduced-to-absent expression of these proteins, consistent with the notion that DNA repair proteins are downregulated during mitotic division.

An intriguing question to be addressed in future studies pertains to what induces the formation of astrocytes with micronuclei in glioblastoma, or in demyelinating disease. The fact that the morphologically identical cells are observed in demyelinating and neoplastic disease, and the absence of a single recurring mutation across all cases, argues against the idea that somatic mutations are responsible for micronuclei formation in Creutzfeldt astrocytes. One possibility is that aneugens (substances that leads to aneuploidy) and clastogens (substances that lead to chromosomal rearrangements) may lead to micronuclei formation (7, 18) in both demyelinating disease and glioblastoma. Further, the presence of these cells in glioblastoma may result from secondary (tumor-induced) demyelination. Regardless of their origin, it is important for surgical neuropathologists to recognize that Creutzfeldt astrocytes and granular mitoses do occur in a subset of glioblastomas and may occasionally be quite numerous in a specimen that is mildly hypercellular. To complicate matters further, a rare example of tumefactive demyelination and glioblastoma occurring simultaneously in the same patient has been reported, with the presence of Creutzfeldt astrocytes in a stereotactic brain biopsy used to support the diagnosis of demyelination (17). In our opinion, however, an alternative interpretation may be limited sampling of a glioblastoma containing secondary areas of demyelination. In summary, our results indicate that glioblastomas with Creutzfeldt astrocyte formation are *IDH*- and *TP53*-wildtype with a clinical course characteristic of glioblastoma, *IDH*-wildtype, lack recurrent mutations that would explain this unique morphology, and retain expression of DNA repair and chromatin remodeling proteins. The mechanisms leading to the formation of these unique cells in both glioblastoma and demyelination is an area in need of further investigation.

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